SCREENING AND IMMOBILIZATION OF HOOGHLY RIVER-BORNE BACTERIA FOR NITROGEN AND PHOSPHORUS NUTRIENTS REMOVAL FROM WASTE WATER

Abstract

Hooghly river water samples from ten different sites were tested for pH, nitrate and orthophosphate, total microbial load and MPN index. Alarming MPN index showed by the samples where excessively high phosphate and nitrate content were recorded, i.e. from Belur, Bishnupur, Garia. High microbial load with good number of phosphate solubilizing bacteria (PSB) was attributed by Dimond harbour and Howrah samples. However, most efficient strain BP02 with high phosphate solubilizing index (PSI:0.78) and nitrate reducing capacity (NRC) had been isolated from Bishnupur water sample. On the basis of morphology and biochemical features, the strain was tentatively grouped as a member of Pseudomonads. The strain BP02 attained its peak for phosphatase enzyme synthesis at 35h of its growth (stationary phase) using inoculum 2% (v/v). Experiments for pH and temperature optima determination with crude enzyme (culture supernatant) showed pH 4.0 and 30^0C is the most suitable conditions for its activity. Freshly grown cells of strain BP02 was taken for immobilization in calcium alginate bead. Immobilized beads with metabolically active cells of BP02 resulted in high degree of phosphate solubilization, and it is more than 10 units of acid phosphatase when kept in *p*-NPP (10mM) for 10min at 30^0C.One unit of phosphatase enzyme was defined as 0.01 µg/ml of *p*-NP formation from the substrate *p*-NPP per minute. The same set of beads showed NRC with high intensity even for repeated cycles of activity. Therefore, application of immobilized BP02 cells bead for waste treatment could be one of the best stable and eco-friendly alternative.

Keywords: Phosphate solubilizing bacteria, nitrate reducing bacteria, biological waste water treatment.

Authors

Susmita Dalui
Department of Microbiology
Lady Brabourne College
Kolkata West Bengal, India.

Soma Pal Saha
Department of Microbiology
Lady Brabourne College
Kolkata West Bengal, India.
soma.palsaha@ladybrabourne.com

I. INTRODUCTION

Cost-effective and safe waste water treatment is a major issue across the globe. Biological waste water treatments are eco-friendly and are applied to make water clean and safe economically. These technologies use bacteria, protozoa and other microbes to purify waste water. The most appropriate waste water treatment systems will avoid harming environment and human health totally. Apart from the important targets of decrease in different hazardous metals, chemicals and biological oxygen demand (BOD) generating pollutants in waste water, the other major necessities are removal of phosphate and nitrogenous compounds. Parallel to the biodegradable carbon staffs, discharges of phosphorus and nitrogen compounds in environment indirectly influence the enhancement of BOD values of aquatic bodies.

Innumerable pharmaceuticals, food, soap and fertilizer industries and municipal waste discharges increase the BOD load to river Ganges and Hooghly. However, the situation is found partly better since the implementation of Ganga Action Plan in 1985. However, after primary treatment (physical and mechanical screening) and several authenticated advanced secondary treatments (mostly biological) the effluents still may consist residual BOD chemicals as suspended solids, organic and inorganic phosphorus and nitrogen compounds which are mostly overlooked by the industries. Aerobic biological phosphate removal is an important process has gained worldwide attention and widely used for phosphorus removal from wastewater [1]. A wide variety of bacterial genus like Bacillus, Pseudomonas, Entero bacter, Rhizobium, Klebsiella, Flavobact erium, Micrococcus showed efficient phosphate solubilizing property isolated from River Ganga [1,2], *Serratia sp*. from River Mahanadi [3], *Pseudomonas sp.* and *Pantoea sp.* from river Shanxi of China [4]. Phosphate solubilizing bacteria(PSB) results in solubilization of different forms of inorganic phosphates by either organic acid synthesis and secretion to external environment or by chelation and exchange reactions in periplasm too[5, 6].

River like Ganges has already been alarmed as a reservoir of nitrates [7,8]. Cleaning technique of nitrogenous compounds by nitrate reducing and denitrifying bacteria as the biological treatment of wastewaters could be promising [9, 10] because of its easy approach. Apart from several water borne pathogenic bacteria, nitrate reduction capacity (NRC) has also been reported by microbes in wetland and estuarine actinobacteria and proteobacteria [11].Therefore, to meet this worldwide challenge in cost effective manner, biological rather bacteriological removal of bulk or residual phosphate and nitrate or nitrite, specially for tertiary treatment of secondary effluent could be the superlative solution.

The present investigation aims at to screen the non-coliform microbial population from Hooghly River water which are efficient in phosphate solubilization and at the same time capable of nitrate reduction and dentrification during its growth phase and to evaluate those characteristics performed by its immobilized cell beads too.

II. MATERIALS AND METHODS

1. **Collection of Water Samples:** For collection of Hooghly River water sample ten different sites from Dakhineswar point to Kakdwip point approx. 110 km (Fig.1) have been chosen during post monsoon season. Pre-sterilized glass containers of 500 ml were

taken for water sampling (triplicate) from 1km distance of river bank. Microbiological experimental work had been initiated on the very day of sample collection and some part was preserved at 10^0C for chemical characters determination.

Figure 1: Water sample collection sites

Fig.1 West Bengal Map (not in scale) showing the range points (**bold arrows**) of Hooghly River where collection was made. Water samples were collected from 10 different sites (triplicate set) and of minimum 15 km apart.

2. **Determination of Biochemical Features of Water Sample:** The collected water samples were tested for their pH, turbidity, contents of orthophosphate and nitrate using pH indicator paper (MERCK), HI MEDIA water testing kit WT015, Orthophosphate testing kit WT008A and Nitrate testing kit WT013, respectively.

3. **Determination of Total Microbial Load and Most Probable Number (MPN) Index For Coliform:** For determination of total microbial load, water sample was serially diluted upto 10^{-6}range followed by spread plate technique on sterile nutrient agar plate (pH 7.2) as per common microbiological technique. Observations were taken after 24h and 48h of incubation at 37^0C. Most probable number of coliforms represents the MPN index per 100 ml of water sample using lactose broth of single strength and double strength and Durhams tube within. Gas production in the Durhams tube after incubation at 37^oC were considered as positive tube and index were recorded using the standard MPN table.

4. **Isolation, Purification and Preservation of Phosphate Solubilizing Bacteria:** For isolation of phosphate solubilizing bacteria, sterile Pikovskaya (PVK) agar (Yeast Extract 0.5 Dextrose, 10.0 Calcium Phosphate, 5.0 Ammonium Sulphate, 0.5 Potassium Chloride, 0.2 Magnesium Sulphate, 0.1 Manganese Sulphate, 0.0001 Ferrous Sulphate, 0.0001 in g/L, pH7.0) plates were used. Dilution tube 10^{-2} and 10^{-4} of water sample were considered as source of inoculum and spread plate technique was followed for isolation of microbes. Plates were incubated for 48h at 37^0C. Colonies with surrounding halo zone were primarily recorded as phosphate solubilizing organisms. To confirm their ability the same strain was transferred thrice to fresh PVK medium and was preserved on PVK slant at

10^0C for further work. The phosphate solubilizing Index (PSI) of the isolated organism was calculated following the formula:
PSI = (Colony diameter, mm + Clear halo zone diameter, mm) / (Colony diameter, mm)

5. **Determination of Nitrate Reduction by the Bacterial Isolates*:*** All PVK grown bacteria were tested for their nitrate reduction ability using Nitrate broth medium containing Trypti case 20.0, Disodium phosphate 2.0, Dextrose 1.0, Agar 1.0, Potassium nitrate 1.0 in g/L (pH 7.0). 5ml of broth in each culture tube was taken for inoculation of bacterial strain. After 24h of incubation growth of organism was recorded and then 6-8 drops of nitrate reagent A (sulfanilic acid 8 g/L in 5M acetic acid) and 6-8 drops of nitrate reagent B (α-napthylamine 5g/L in 5M acetic acid) were added to the tube and were compared with similarly treated control blank tube without microorganism. Colour development was noted. If no colour developed, zinc powder was added for chemical reduction process. Observation was recorded after at least 3 minutes for a red colour to develop after addition of zinc powder. Positive Test showed development of cherry red coloration on addition of reagent A and reagent B; Negative Test showed no colour change and development of red colour on adding zinc powder.

6. **Characterization of Bacterial Strains:** On the basis of phosphate solubilization and nitrate reduction capacity most potent organism had been selected. The selected bacterial strain was characterized by their colony morphology, gram stain responds, cell shape, sporulation, extracellulular polysachharide (EPS) production etc. followed by the standard microbiological staining procedures. Gram character was also confirmed using 3% (w/v) KOH soln. Carbon source utilization pattern of the potent strains had also been determined using glucose, fructose, maltose, sucrose, lactose, cellobiose, cellulose and starch as sole source of carbon following streak plate method on individual solid agar plate.

7. **Preparation of Para-Nitrophenol Phosphate (*P*-NP) Standard Curve:** Standard curve of *p*-NP was prepared using Para-nitrophenol (*p*-NP) Sigma, USA, 100µM as stock and NaOH (0.1N) as reagent. 1-100µM of *p*-NP were prepared, incubated for 15min at 37^0C and optical density was recorded at 420nm wavelength using Spectrophotometer A124256.Values of optical density were plotted against the known concentration to derive the standard curve of *p*-NP.

8. **Assay of Phosphatase Enzyme :** Assay of phosphatase was performed as described by Tabatabai and Bremmer (1968) [12]. Fresh culture of bacterial strain was taken for centrifugation (10000 x g for 10 min at 5^0C) and the culture supernatant had been used as crude enzyme source. *Para*-nitrophenyl phosphate (*p*-NPP) at 10mM was taken as substrate. One unit of phosphatase enzyme was defined as 0.01 µg/ml of *p*-NP formation from the substrate *p*-NPP per minute.

9. **Determination of Optimized Cultural Condition for Growth and Phosphatase Enzyme Synthesis:** Growth measurement and concomitant enzyme synthesis by the potent isolate were performed at 5 hr interval. The potent strain was cultured with 2% (v/v) inoculums in PVK broth medium under shake flask condition at 37^0C. Growth was recorded as viable cell count per unit volume using haemocytometer. Cell free

supernatant was taken after centrifugation (10000 x g) as crude enzyme source. The optimum dose of inoculum (v/v) has been determined using 1-4% inoculum in PVK broth culture. Growth and phosphatase enzyme synthesis by the organism were determined after 24h and 48h of incubation period.

10. **Determination of pH And Temperature Optima of Crude Enzyme:** For determination of pH optima of crude enzyme, buffers (0.1M) of variable pH had been used, these were: sodium acetate buffer (pH3), sodium acetate buffer(pH4), citrate buffer (pH5& 6), saline acetate buffer (pH7), glycine-NaOH buffer (pH8). Using the buffer of optimum pH, crude enzyme activity was tested at different temperature ranging between 25^0C-45^0C following the same protocol.

11. **Immobilization of Whole Cell of Potent Strain:** Immobilized calcium alginate beads of potent strain had been prepared using 20ml of 4% (w/v) Na-alginate (SRL) gel, 0.4g of fresh log phase bacterial cells from PVKA medium (scrapped and washed aseptically) and 50ml calcium chloride soln. (0.1M). Beads were incubated in fresh $CaCl_2$ soln. At 10^0C for 2h to get high compact beads. Sterile water washed beads were tested for phosphatase activity and nitrate reduction capacity.

III. RESULTS AND DISCUSSION

1. **Determination of Physico-Chemical Characterization of Collected Water Sample:** The pH value of the water samples collected from ten different sites Belur, Bishnupur, Canning, Diamond harbour, Dakhineswar, Garia, Howrah, Kakdwip, Kalighat, Kulpi showed almost slightly acidic to neutral. Turbidity of the samples appeared good (10-25NTU) except the samples of Kalighat, Diamond harbour, Belur, Bishnupur which were more than 25 NTU. The nitrate content of the sample ranged between 1mg/L -45mg/L, higher values were present in the samples of Howrah and Kulpi. Most of the waters showed the orthophosphate content less than 10mg/L except from Garia and Howrah. All the results are the average of triplicate samples and represented in the Table 1.Typically, standard nitrate level for drinking water is 3-3.7mg/L and for an aquatic body 10mg/L (WHO, 1999), however, samples from Belur, Bishnupur, Kulpi and Howrah exceed that limit. All samples showed extremely high phosphate too (prescribed limit is 1mg/L).

Table1: **Physico-chemical features of water samples**

Collection site	Coordinate	pH	Turbidity, NTU	Nitrogen as nitrate, mg/L	Phosphorus as Orthophosphate, mg/L
Belur	22.6378°N, 88.349°E	6.9	>25	30	5
Bishnupur	23.0678 °N, 87.3165°E	7.0	>25	20	8
Canning	22.0678 °N, 88.3954°E	7.5	10	1	3
Diamond harbour	22.193°N, 88.184°E	6.8	>25	2	5

Dakhineswar	22.6575°N, 88.3654°E	6.5	10-25	5	3
Garia	22.2409 °N, 88.2815°E	6.8	10-25	10	15
Howrah	22.5176°N, 88.2654°E	6.8	10	25	20
Kakdwip	21.5957 °N, 88.1996°E	6.5	10-25	1	2
Kalighat	22.519°N, 88.345°E	7.0	>25	8	3
Kulpi	22.0763°N,88.2557 °E	6.3	10-25	45	3

All values represent average of triplicate set of samples

2. **Determination of Total Microbial Load and MPN Index of Collected Water Sample:** The microbial load, CFU/ml of the collected water samples showed the range between 5×10^3 to 77×10^3 and the maximum load was present in Kulpi sample. Presence of coliform in terms of MPN index/100ml ranged between 17 and >2400 and the maximum value was represented by the sample of Belur, Bishnupur and Howrah (Table 2). However, the higher values of microbial load are not very corresponding to that of higher MPN index of the water samples.

Table 2: Microbial load and the MPN index of different water samples

Collection site	Range of microbial Load on NA[#]: CFU/ml	Coliform[#]: MPN index/100ml
Belur	19×10^3 -24×10^3	>2400
Bishnupur	6×10^3 -8×10^3	>2400
Canning	4×10^3 - 5×10^3	26
Diamond harbour	49×10^3 - 65×10^3	350
Dakhineswar	18×10^3 - 24×10^3	280
Garia	3×10^3 - 5×10^3	94
Howrah	55×10^3 -62×10^3	>2400
Kakdwip	29×10^3 - 38×10^3	17
Kalighat	3×10^3 - 6×10^3	45
Kulpi	61×10^3-77×10^3	110

[#] All values after 24h incubation at 37^0C; MPN index appeared same for all sets from same site

3. **Isolation of Phosphate Solubilizing Bacteria (PSB):** Water sample (three samples of each site) was diluted upto 10^{-3} dilution and was placed on PVK agar. Almost all collected samples except from Dakhineswar showed the presence of PVKA growing and phosphate solubilising bacteria. Not each and every PVK growing organisms showed the visible efficiency of phosphate solubilization (Table 3). More than 60% of isolated strains from Diamond horbour showed phosphate solubilizing efficiency. Colony with different

morphology was designated with individual strain number. The colonies showing the good visible solubilization zone was only taken for nitrate reduction capacity (NRC) test.

Table 3: Determination of the Phosphate solubilizing index (PSI) by the colonies on PVK

Collection site	Range of CFU/ml on PVKA *	%, PSB strain with visible phosphate solubilization zone*#
Belur	15×10^3 - $17x10^3$	56.52 ± 2.8
Bishnupur	4×10^3 - 7 $x10^3$	32.5 ± 1.5
Canning	1×10^3-2 $x10^3$	22.4 ± 1.8
Diamond harbour	21×10^3 - 25 x 10^3	60 ± 3.5
Dakhineswar	No colony found	NA
Garia	0-1×10^3	10.68 ± 0.7
Howrah	18 x 10^3 - 24×10^3	50.45 ± 2.5
Kakdwip	1×10^3 -3 $x10^3$	20.65 ± 1.5
Kalighat	9 $x10^3$ - 13×10^3	41.58 ± 3.1
Kulpi	4×10^3-7 x 10^3	30.7 ± 2.2

*****After 48h of incubation at 37^0C; #Data derived for triplicate water samples from same site; NA

4. **Determination of PSI and NRC By The Selected Strains:** The colonies with good phosphate solubilization zone were selected and were taken for nitrate reduction capacity test. Strain KG01 showed PSI as high as 0.86 followed by strain DH02 and BP02 with PSI 0.83 and 0.73, respectively (Table 4). As the bacterial strain BP02 had resulted in high and early NRC, it has been selected as the most potent candidate for further experiments. The strain BP02 was further tested for presence of residual nitrite after 48h and was found negative result with ammonia smell, suggesting media nitrates were reduced beyond nitrites to ammonia or might be with nitrogen gas.

Table 4: Selection of potent strain on the basis of PSI and NRC

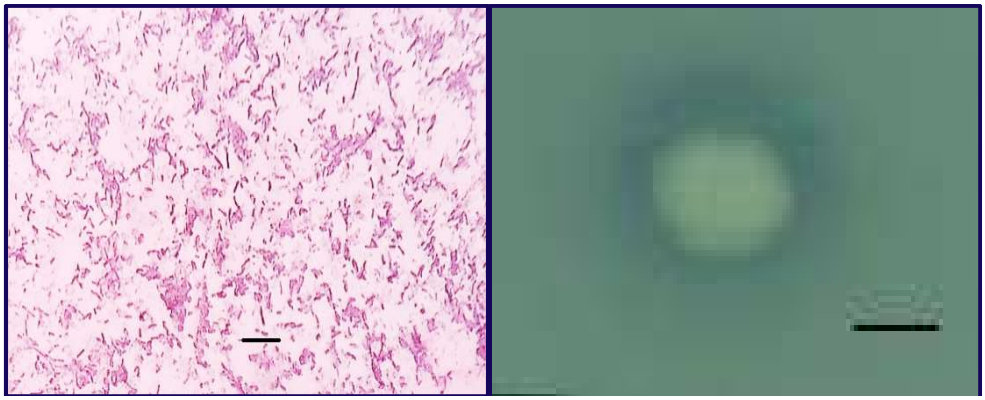
Collection site	Strains	PSI#		NRC *		Remark on NRC*
		24h	48h	24h	48h	
Belur	BM01	0.625	0.65	-	+	Low and late
Belur	BM02	0.166	0.33	-	-	Negative
Belur	BM03	0.5	0.416	-	-	Negative
Bishnupur	BP01	0.33	0.33	-	-	Negative
Bishnupur	BP02	0.72	0.78	++	+	High and early
Canning	CN01	0.083	0.084	+	+	Moderate and consistent

Diamond harbour	DH01	0.13	0.55	++	-	High and early
Diamond Harbour	DH02	0.66	0.83	-	-	Negative
Dakhineswar	DS01	0.33	0.2	-	-	Negative
Garia	GR01	0.245	0.14	-	-	Negative
Garia	GR02	0.37	0.183	+	+	Moderate and consistent
Howrah	HW01	0.775	0.71	-	-	Negative
Kakdwip	KD01	0.1	0.11	++	-	High and early
Kalighat	KG01	0.5	0.86	-	+	Low and late
Kalighat	KG02	0.3	0.15	-	-	Negative

#Average value of triplicate sets of plate cultures
*On the basis of colour intensity after reagent sulfanilic acid and α-napthylamineaddition

5. **Characterization of Potent Bacterial Strain BP02:** Considering both phosphate solubilizing efficiency and nitrate reduction capacity the strain BP02 isolated from Bishnupur site of Hooghly River water had been selected as the potent isolate and was taken for successive experiments. The potent strain BP02 had been characterized with white medium sized regular colony (3.1mm diameter), gram negative, aerobic, motile, non-capsulated and non-sporulatinglong bacilli, showed high intensity of growth on glucose, lactose, maltose, sucrose, cellobiose, medium intensity on starch and cellulose and poor to no growth on fructose, mannitol and glycerol containing plates. The organism did not ferment and produce gas during growth in glucose and lactose broth and it appeared as non-coliform. On the basis of morphology of colony and cells and biochemical cultural behaviour and as per description in Bergey's Manual of Determinative Bacteriology (9^{th} ed.) [13] ,it is tentatively identified as a strain under Pseudomonads.

Plate1: A. Strain BP02 after gram staining (Bar represents 5µm) B. Strain BP02 colony on PVKA with halo zone of solubilization of calcium phosphate surrounding the colony (Bar represents 3mm).



6. **Determination of Inoculums Dose For Optimum Enzyme Synthesis:** Inoculum dose as 1-4% (v/v) individually was used to PVK broth medium and was allowed to grow for 24h and 48h for determination of growth (cells /ml) and phosphatase enzyme synthesis. In every case pH of the culture supernatant dropped by around pH 4.8. Hence, the enzyme assay was performed using citrate buffer of pH 5.0. Growth in terms of cells per volume increased with time and enhanced dose of inoculum, however, maximum enzyme synthesis happened with 2% inoculum but it decreased in 48h of growth (Table 5**).** Decrease in extracellular enzyme phosphatise in late hours might be due to rapid utilization of carbon and other media ingredients for heavy biomass formation and presence of active enzyme in the growth environment.

Table 5: Determination of optimum inoculum dose for growth and phosphatise enzyme synthesis of by strain BP02

Inoculums dose, %, (v/v)	24h		48h	
	Cells x 10^5/ml	Enzyme, unit/ml	Cells x 10^5/ml	Enzyme, unit/ml
1	8.5 ±0.23	10.09 ±0.06	17.5 ± 0.7	3.97± 0.03
2	14 ±0.8	13.15 ± 0.1	19 ± 1.1	7.18± 0.02
3	18.5 ±1.2	11.93 ± 0.13	29.5 ± 0.9	4.89± 0.022
4	20.5 ±1.1	5.37 ± 0.03	33.5 ± 1.6	5.50±0.016

1 unit of phosphatase enzyme is equivalent to 0.01 µg/ml of *p*-NP formation ability from the substrate *p*-NPP (10mM) per minute in citrate buffer (pH 5.0)

7. **Determination of Growth and Activity of Phosphatase Enzyme by the Potent Strain BP02:** Growth and concomitant enzyme synthesis by the strain BP02 had been performed using PVK broth medium. Maximum growth in terms of cell no./ml was found at around 40h of incubation; however, from curve it had been predicted that stationary phase started from 35h. Maximum phosphatase enzyme 3.9 unit/ml was found at 35h when the pH of the culture supernatant dropped by pH 4.8 (not shown in figure). Hence, the enzyme assay was performed using citrate buffer of pH 5.0. Fig.1 represents time courses of growth and enzyme synthesis by the strain BP02. Reduction of enzyme synthesis during stationary phase of growth might be due to the sufficient amount of soluble phosphate as no insoluble granular phosphate was visible in medium.

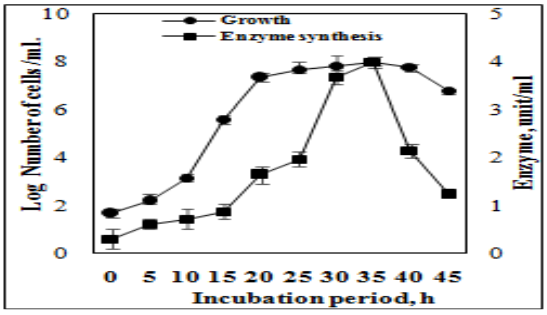


Figure 1: Time course of growth and phosphatase enzyme synthesis by Strain BP02 Incubation of organism was done under shake flask condition (140rpm) at 37^0C for total duration.

8. **Determination of pH and Temperature Optima Of Phosphatase Enzyme Synthesized By Strain BP02:** The pH and temperature optima were determined from culture supernatant of strain BP02 grown in PVK broth up to 35h. Buffers of different pH (pH 4- pH8) were used for the enzyme assay and the maximum enzyme activity was found at pH 4.0 where enzyme activity was 9.7 unit/ml (Fig 2A). With increase in pH value of assay reaction medium the activity dropped sharply. The maximum enzyme activity using pH4.0 (acetate buffer) was found when the assay was performed at 30^0C; below and above this temp. enzyme activity declined and appeared nil at 20^0C (Fig.2B). Hence, the enzyme synthesized by the strain appeared as acid phosphatase. Synthesis of extracellular acid phosphatase enzyme is a common phenomenon by several pathogenic strains. However, several non-pathogenic strains like *Burkholderiasp. [14],* and Pseudomonas sp. [15] have been reported with acid *phosphatase. Burkholderia sp. from agricultural land of Brazil [14] and Serratia* sp. from Mahanadi River delta [3] showed very good acid phosphatase response for solubilization and mineralization of inorganic phosphates of soil.

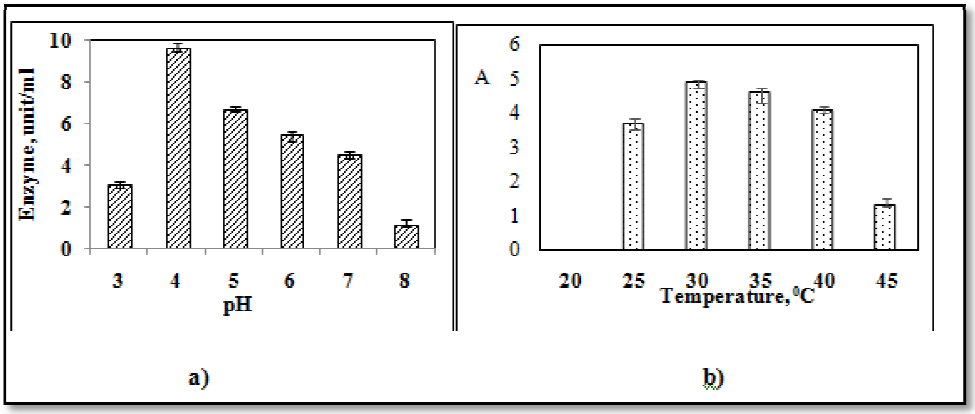


Figure 2: Determination of pH (a) and temperature (b) optima for phosphatase enzyme of strain BP02 Incubation of organism was done under shake flask condition (140rpm) for 35h.

9. **Determination of Phosphatase Enzyme Efficiency and Nitrate Reduction Capacity By The Immobilized Cells Of Strain BP02 :** Freshly grown (35h) strain BP02 culture on PVK agar plate was taken for high cell density immobilized bead preparation. To check the activity of immobilized BP02 strain cells (Plate 2), fresh beads in different number (1-5) were taken in 5ml of *p*-NPP soln. of 10mM strength and incubated for 10 min, the resultant was assayed for *p*-NP quantification following the previously described method of enzyme assay. With increase in number of beads the reaction mixture showed enhanced enzyme activity (Fig.3). The same sets of washed beads also were used for checking of their nitrate reduction capacity and the enhanced capacity were recorded with increased number of beads (Plate 3). Thus, calcium alginate beads containing cells of strain BP02 could be very promising in dual function of bioremediation, mobilization of insoluble inorganic calcium phosphate and removal of nitrate following nitrate reduction and denitrification finally. The beads efficiency was justified using the same beads up to 4^{th} round application for phosphatase and nitrate reduction as well (Table 6). Acid Phosphat ase activity declined by 20% approx. from 3^{rd} cycle, however, nitrate reduction ability of the beads decreased slowly.

Plate 2: Freshly prepared calcium alginate beads of strain BP02 cells

Beads were of 2.5mm (approx.) in diameter and preserved in sterile saline water at 10^0C.

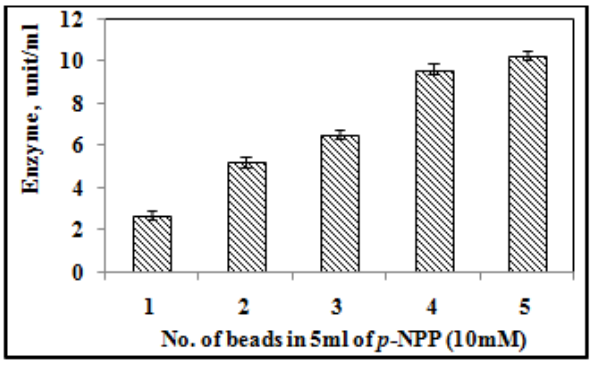
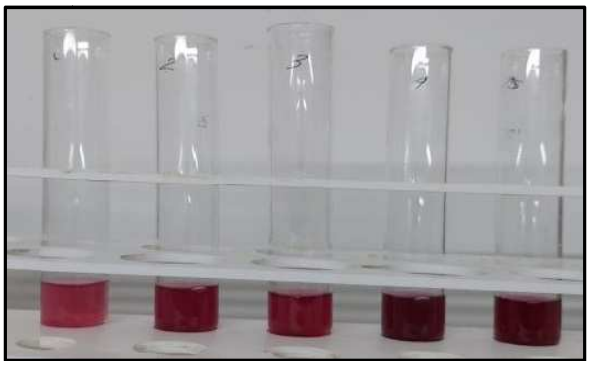


Figure 3: Phosphatase enzyme activity of immobilized cells of strain BP02

Beads in *p*-NPP (10mM) were kept under static condition for 10min at 30^0C and 0.1ml of the reaction mixture was taken for enzyme assay.

Plate 3: Nitrate reduction capacity of same immobilized cells of BP02



1-5 beads(from left to right) were incubated in 5ml of sterile nitrate broth for 10min and the whole reaction mixture of individual tubes was taken for nitrate reduction assay. Beads-free broth showing the increased colour intensity developed.

Table 6: Comparative efficiency of BP02 cells beads in recycling application

Cycle	Acid Phosphatase activity	Nitrate reduction capacity
1st	+++	+++
2nd	+++	+++
3rd	++	+++
4th	++	++

The 1st cycle efficiency was considered as 100%

Ideas for application of immobilized cells beads for treatment of different industrial effluent have been developed in last two decade only. Use of microalga like *Chlorella* achieved popularity for nutrient pollutants removal from waste water [16,1718; 19]. However, limited reports on use of bacterial cells beads are present, applied for waste water treatment, e.g- advanced municipal wastewater treatment by *Azospirillum brasilense* with chlorella immobilized in alginate [20], removal of methylene blue by Bacillus paramycoides, where cells are immobilized on cellulose acetate-polyethylene oxide nanofibers[21], ammonia oxidation by archeal hydrogel beads [22] and bioremediation of textile dyes by activated sludge organisms immobilized in alginate [23].

IV. CONCLUSION

In this investigation significant microbial load was found in Hooghly River. Apart from presence of coliform, water samples harboured bacterial strains with effective phosphate solubilizing ability and nitrate reducing capacity. The potent bacterial strain BP02, tentatively identified as a member of *Pseudomonas* which showed extracellular acid phosphatase enzyme synthesis during its stationary phase and able to reduce nitrates at the same time. A successful attempt of immobilized calcium alginate beads of BP02 cells exhibited promising results of acid phosphate ase activity and nitrate reduction capability within very short period of time and for repeated cycle use too. This advanced technology of waste water treatment using indigenous aquatic bacterial strain instead of conventional chemical processes might be the best adopted recyclable method to achieve the clean environment. After proper identification and more essential thorough experiments with immobilized bead in polluted water samples, this non-coliform bacterial strain can be exploited as a commercially effective tool for low-cost and eco-friendly waste water treatment.

REFERENCES

[1] D Paul and S. N. Sinha, “Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river Ganga, India,”Annals of Agrarian Science, vol.15(1) pp. 130-136, 2017,https://doi.org/10.1016/j.aasci.2016.10.001.

[2] D.Paul and S. N. Sinha, “Isolation of phosphate solubilizing bacteria and total heterotrophic bacteria from river water and study of phosphatase activity of phosphate solubilizing bacteria,”Advances in Applied Science Research, vol. 4(4) pp. 409-412,2013 ISSN: 0976-8610 CO.

[3] B.C. Behera, H. Yadav, S.K. Singh, R.R. Mishra, B.K. Sethi, S.K. Dutta, and H.N. Thatoi, “Phosphate solubilization and acid phosphatase activity of *Serratia sp*. isolated from mangrove soil of Mahanadi

River delta, Odisha, India," Journal of Genetic Engineering and Biotechnology, vol. 15(1), pp. 169-178, 2017 ISSN 1687-157X, https://doi.org/10.1016/j.jgeb.2017.01.003.

[4] Q. Chen and S. Liu,"Identification and Characterization of the phosphate solubilizing bacterium *Pantoeasp.S32* in reclamation soil inShanxi, China," FrontMicrobiol.,vol.10, 2019, https://www.frontiersin.org/articles/10.3389/fmicb.2019.02171

[5] S.B. Sharma, R.Z. Sayyed, M.H. Trivedi, and T.A. Gobi, "Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils," Springer Plus, vol. 2, pp.587-600, 2013, https://doi.org/10.1186/2193-1801-2-587.

[6] T. A. Elizabeth,R. G. Bernard, and O. O. Babalola, "Microbial phosphorus solubilization and its potential for use in sustainable agriculture," Front Microbiol., Sec. Plant Pathogen Interactions,vol. 8, 2017,https://doi.org/10.3389/fmicb.2017.00971.

[7] R Prasad andS Prasad,"Algal blooms and phosphate eutrophication of inland water ecosystems with special reference to India,"International Journal of Plant and Environment, vol.5, 2019. 10.18811/ijpen.v5i01.1.

[8] A Kumar, A. Ajay,B., Dasgupta, P. Bhadury, and P Sanyal, "Deciphering the nitrate sources and processes in the Ganga river using dual isotopes of nitrate and Bayesian mixing model," Environmental Research,vol216 (4),2023.https://doi.org/10.1016/j.envres.2022.114744.

[9] Rossi, O. Motta, S. Matrella., A. Proto, and G. Vigliotta,"Nitrate removal from wastewater through biological denitrification with OGA 24 in a batch reactor,"Water, vol. *7*(1), pp.51-62; 2015, https://doi.org/10.3390/w7010051.

[10] Z. Chen, T.Zhang, J. Meng, S. Zhou, Z. Zhang, Z. Chen, Y. Liu, J. Zhang, and J. Cui,"Efficient nitrate removal of immobilized mixed aerobic denitrifying bacteria and community dynamics response to temperature and low carbon/nitrogen polluted water," Bioresource Technology, vol. 362, pp.127873,2022 https://doi.org/10.1016/j.biortech.2022.127873.

[11] M.Khajah, F. Bydalek, A. O. Babatunde, A. Al-Matouq, J.Wenk, and G.Webster,"Nitrogen removal performance and bacterial community analysis of a multistage step feeding tidal flow constructed wetland," Front. Water, Sec. Water and Built Environment, vol. 5, 2023, https://doi.org/103389/frwa.1128901.

[12] M., Tabatabai and J. Bremmer,"Use of *p*-nitrophenylphosphate for assay of soil phosphatase activity," Soil Biology and Biochemistry, vol.1, pp. 301–307, 1969.

[13] Bergey's Manual of Determinative Bacteriology.9th Edition- 9 October 1993. ISBN-13: 978-0683006032 ISBN-10: 9780683006032.

[14] T.H. Rombola, E.A. Pedrinho, E.G. de Macedo Lemos, A.M. Gonçalves, L.F. dos Santos, and J.M. Pizauro,"Identification and enzymatic characterization of acid phosphatase from *Burkholderia gladioli*," BMC Research Notes. vol.7pp.221, Apr 9; 2014. doi: 10.1186/1756-0500-7-221.

[15] W. Song, K. Zheng, X. Xin & G. Cong, L. Guo, L. Jia, C. Xiulai, L. Jie,and H.Guipeng,"Enzymatic production of ascorbic acid-2-phosphate by engineered *Pseudomonas aeruginosa*acid phosphatase," Journal of Agricultural and Food Chemistry,vol.69., 2021,10.1021/acs.jafc.1c04685.

[16] S.Chen-Lin, C. Cheng-Ann, O.Bojo, and H. Yii-Siang,"Feasibility of marine microalgae immobilization in alginate bead for marine water treatment: bead stability, cell growth, and ammonia removal," International journal of Polymer Science,vol. 2017, Article ID6951212https://doi.org/10.1155/2017/6951212

[17] Z. Bouabidi,M. El-Naas,and Z.Zhien,"Immobilization of microbial cells for the biotreatment of wastewater: A review," Environmental Chemistry Letters, vol. 17, 2018,10.1007/s10311-018-0795-7.

[18] Q. Emparan, H. Razif,andJ.Yew,"Phycoremediation of treated palm oil mill effluent (TPOME) using *Nannochloropsis sp*. cells immobilized in the biological sodium alginate beads: effect of POME concentration," Bioresources,vol.14, pp. 9429-9443. 2019,10.15376/biores.14.4.9429-9443.

[19] A.Alhumairi, R. Hamouda, and A. Saddiq,"Comparative study between immobilized and suspended *Chlorella sp* in treatment of pollutant sites in Dhiba port Kingdom of Saudi Arabia,". Heliyon,vol. 8 (9),September 2022, e10766. https://doi.org/10.1016/j.heliyon.2022.e10766.

[20] S.A. Covarrubias, L.E. de-Bashan, M. Moreno, andY. Bashan,"Alginate beads provide a beneficial physical barrier against native microorganisms in wastewater treated with immobilized bacteria and microalgae," Applied Microbiology and Biotechnology,vol. 93(6), pp. 2669-2680,Mar;2012,doi: 10.1007/s00253-011-3585-8. Epub 2011 Oct 27. PMID: 22038243.

[21] D. Zamel, A.H. Hassanin, R. Ellethy, G. Singer, andA. Abdelmoneim,"Novel bacteria-immobilized cellulose acetate/poly(ethylene oxide) nanofibrous membrane for wastewater treatment," Science Report, vol. 12;9(1) pp. 18994,Dec 2019, doi: 10.1038/s41598-019-55265-w.

[22] Landreau, H. You, and D.A Stahl, “Immobilization of active ammonia-oxidizing archaea in hydrogel beads,”Clean Water, vol.4, pp.43, 2021,https://doi.org/10.1038/s41545-021-00134-1

[23] Y. A. Bustos-Terrones, E. R. Bandala, G. E. Moeller-Chávez, and V. Bustos-Terrones,“Enhanced biological wastewater treatment using sodium alginate-immobilized microorganisms in a fluidized bed reactor,” Water Science and Engineering, vol. 15 (2)pp. 125-133, 2022, https://doi.org/10.1016/j.wse.2022.02.002.

